GFP-VIVIT expression vector contained an oligonucleotide coding for MAGPHPVIVITGPHEE at the NH_2-terminus of GFP. Twenty-four hours after transfection, cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA) (20 nM) and ionomycin (1 μM) or with immobilized anti-CD3 (0.2 μg/ml) (HIT3a, Pharmingen) and soluble anti-CD28 (0.5 μg/ml) (CD28.2, Pharmingen). CsA was added 30 min before the stimuli. Luciferase activity in cell lysates was normalized to levels of hGH.

12. The calcineurin targeting site of murine NFAT1, GPSPRIEITPSHELMQAGG (residues 108 through 126) was mutated to GPHPVIVITGPHELMQAGG (substitutions underlined) by replacing the DNA encoding the wild-type NFAT1 sequence between flanking Bsp 120I and Eco O109I sites with an oligonucleotide encoding the mutant sequence. Dephosphorylation of HA-NFAT1-GFP (*9*) was assessed in whole-cell extracts by protein immunoblotting, and subcellular localization was analyzed by GFP fluorescence (*5*).
13. Jurkat cells cotransfected with murine CD4 (mCD4) (0.75 mg per 10^6 cells) and GFP or GFP-VIVIT (0.75 μg per 10^6 cells) were selected with magnetic beads coated with anti-mCD4 (Dynabeads L3T4, Dynal, Lake Success, NY) (*22*). The selected mCD4-expressing cells were >90% GFP- or GFP-VIVIT–positive, whereas mCD4-nonexpressing cells were <5% GFP-positive, as assessed by fluorescence microscopy. Multiprobe RNase protection assays were performed with the RiboQuant multiprobe kit (Pharmingen) (*22*).
14. A. M. Ranger *et al.*, *Nature* **392**, 186 (1998); J. L. de la Pompa *et al.*, *ibid.*, p. 182.
15. I.-C. Ho, J. H.-J. Kim, J. W. Rooney, B. M. Spiegelman, L. H. Glimcher, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15537 (1998).
16. A. C. Greenlund *et al.*, *Immunity* **2**, 677 (1995); U. Schindler, P. Wu, M. Rothe, M. Brasseur, S. L. McKnight, *ibid.*, p. 689; T. Tsukazaki, T. A. Chiang, A. F. Davison, L. Attisano, J. L. Wrana, *Cell* **95**, 779 (1998); L. Attisano and J. L. Wrana, *Curr. Opin. Cell Biol.* **10**, 188 (1998); T. Kallunki, T. Deng, M. Hibi, M. Karin, *Cell* **87**, 929 (1996); A. J. Whitmarsh, J. Cavanagh, C. Tournier, J. Yasuda, R. J. Davis, *Science* **281**, 1671 (1998).
17. N. H. Sigal *et al.*, *J. Exp. Med.* **173**, 619 (1991); M. Pascual *et al.*, *Immunol. Today* **11**, 514 (1998). M. Hojo *et al.*, *Nature* **397**, 530 (1999); G. J. Nabel, *ibid.*, p. 471; M. J. Mihatsch *et al.*, *Clin. Nephrol.* **49**, 356 (1998); M. Cecka, *Surg. Clin. North. Am.* **78**, 133 (1998).
18. C. Loh *et al.*, *J. Biol. Chem.* **271**, 10884 (1996).
19. C. Luo *et al.*, *Mol. Cell. Biol.* **16**, 3955 (1996).
20. F. Mercurio *et al.*, *Science* **278**, 860 (1997).
21. J. P. Northrop *et al.*, *Nature* **369**, 497 (1994); T. Hoey, Y. L. Sun, K. Williamson, X. Xu, *Immunity* **2**, 461 (1995).
22. A. Kiani, J. P. B. Viola, A. H. Lichtman, A. Rao, *Immunity* **7**, 849 (1997).
23. We thank M. Berne for peptide synthesis and sequencing; D. Littman, G. Crabtree, and T. Hoey for reagents; and members of the laboratory for helpful discussion and advice. Supported by NIH grants R01 AI 40127 (to A.R.), R43 AI 43726 (to P.G.H.), R01 HL 03601 (to M.B.Y.), and R01 GM 56203 (to L.C.C.); by the Ministerio de Educación y Ciencia, Spain (C.L.-R.); and by the Arthritis Foundation (J.A.).

3 June 1999; accepted 24 August 1999

Identification of an RNA-Protein Bridge Spanning the Ribosomal Subunit Interface

Gloria M. Culver,* Jamie H. Cate,† G. Zh. Yusupova, Marat M. Yusupov, Harry F. Noller‡

The 7.8 angstrom crystal structure of the 70*S* ribosome reveals a discrete double-helical bridge (B4) that projects from the 50*S* subunit, making contact with the 30*S* subunit. Preliminary modeling studies localized its contact site, near the bottom of the platform, to the binding site for ribosomal protein S15. Directed hydroxyl radical probing from iron(II) tethered to S15 specifically cleaved nucleotides in the 715 loop of domain II of 23*S* ribosomal RNA, one of the known sites in 23*S* ribosomal RNA that are footprinted by the 30*S* subunit. Reconstitution studies show that protection of the 715 loop, but none of the other 30*S*-dependent protections, is correlated with the presence of S15 in the 30*S* subunit. The 715 loop is specifically protected by binding free S15 to 50*S* subunits. Moreover, the previously determined structure of a homologous stem-loop from U2 small nuclear RNA fits closely to the electron density of the bridge.

Ribosomes are large ribonucleoprotein complexes that are responsible for the fundamental process of protein synthesis. They are composed of two asymmetric subunits, each of which contributes to specific functions during translation. The interface between these subunits allows for the coordination of these discrete functions and also provides the binding surfaces for many substrates and ligands. Thus, the identification of specific molecular interactions between the two subunits is of great importance. Numerous experiments have identified RNA and protein elements that potentially contribute to this subunit-subunit interface (*1–3*). However, in the absence of high-resolution structural information, identification of the molecular components comprising specific subunit-subunit interactions has been difficult.

The 7.8 Å x-ray crystal structure of the *Thermus thermophilus* 70*S* ribosome (*4*) shows that the two ribosomal subunits are connected by a complex network of molecular interactions. One of these (bridge B4) can be identified as a double-stranded RNA stem-loop that is continuous with the 50*S* subunit and makes contact with the bottom of the platform of the 30*S* subunit (Fig. 1). Immunoelectron microscopy and preliminary modeling studies of the 30*S* subunit based on extensive biochemical, biophysical, and phylogenetic evidence localize the binding site for protein S15 to this region of the 30*S* subunit (*5*). Additionally, evidence for the placement of S15 at the subunit interface has come from intersubunit cross-linking studies (*2*) and a temperature-sensitive S15 mutant that is defective in subunit association (*6*).

To test the possible proximity of S15 to 23*S* ribosomal RNA (rRNA), we performed directed hydroxyl radical probing (*7*). Iron(II) was tethered by a linker, 1-(*p*-bromoacetamidobenzyl)-EDTA (BABE) (*8*), to unique cysteine (C) residues on the surface of S15 at amino acid positions 12, 36, 46, and 70 by directed mutagenesis (*9*), using the published solution and crystal structures of S15 as a guide (*10*). The Fe(II)-derivatized proteins were incorporated into 30*S* subunits by in vitro reconstitution (*11*) and associated with 50*S* subunits to form 70*S* ribosomes, which were then purified by sucrose gradient centrifugation. The 30*S* subunits containing S15 derivatized at position 70 failed to associate with 50*S* subunits, although they appeared to be normally as-

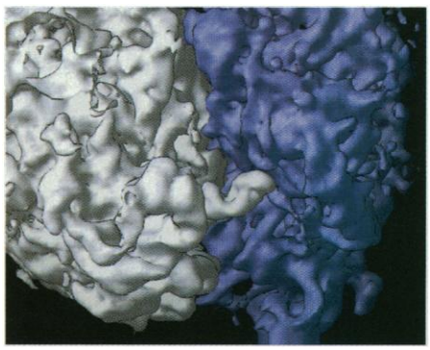

Fig. 1. Electron density from the 7.8 Å crystal structure of the *T. thermophilus* 70*S* ribosome (*4*) showing interaction of a discrete RNA feature of the 50*S* subunit (white) with the bottom of the platform of the 30*S* subunit (blue). Electron density is contoured at 1.1σ.

Center for Molecular Biology of RNA, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA.

*Present address: Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011, USA.

†Present address: Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge, MA 02142, USA.

‡To whom correspondence should be addressed.

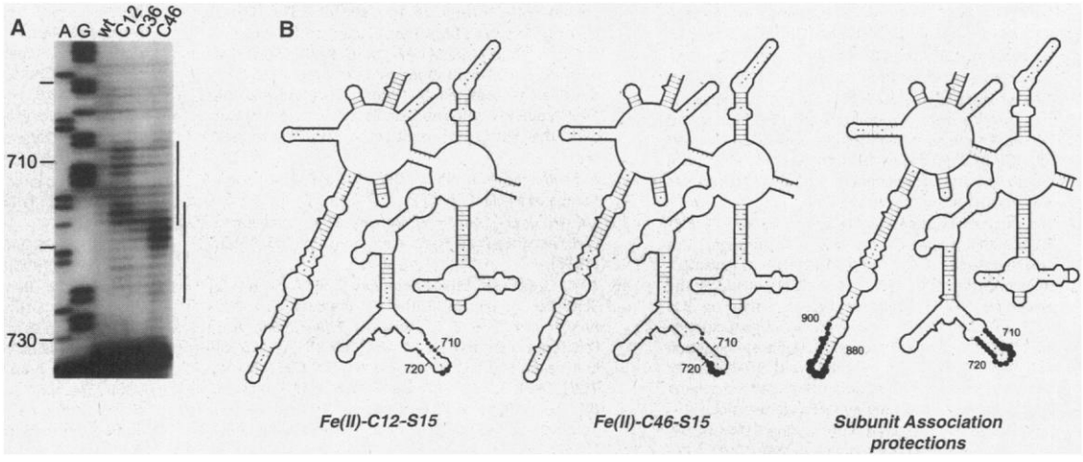

Fig. 2. (**A**) Directed hydroxyl radical cleavage of 23*S* rRNA in 70*S* ribosomes containing Fe(II)-derivatized ribosomal protein S15 in reconstituted 30*S* subunits. Sites of cleavage were localized by primer extension with reverse transcriptase. A and G, sequencing lanes; wt, mock Fe(II)-BABE–treated (cysteine-free) wild-type S15; C12, Fe(II)-C12-S15; C36, Fe(II)-C36-S15; C46, Fe(II)-C46-S15. Vertical lines at the right indicate regions of cleavage. (**B**) Positions of directed hydroxyl radical cleavage from Fe(II)-C12-S15 and Fe(II)-C46-S15. Nucleotides within this region of 23*S* rRNA that are protected by subunit association from free hydroxyl radicals (*3*) are also shown. Intensities of cleavages are indicated by large, medium, or small solid circles (high through low intensity, according to size).

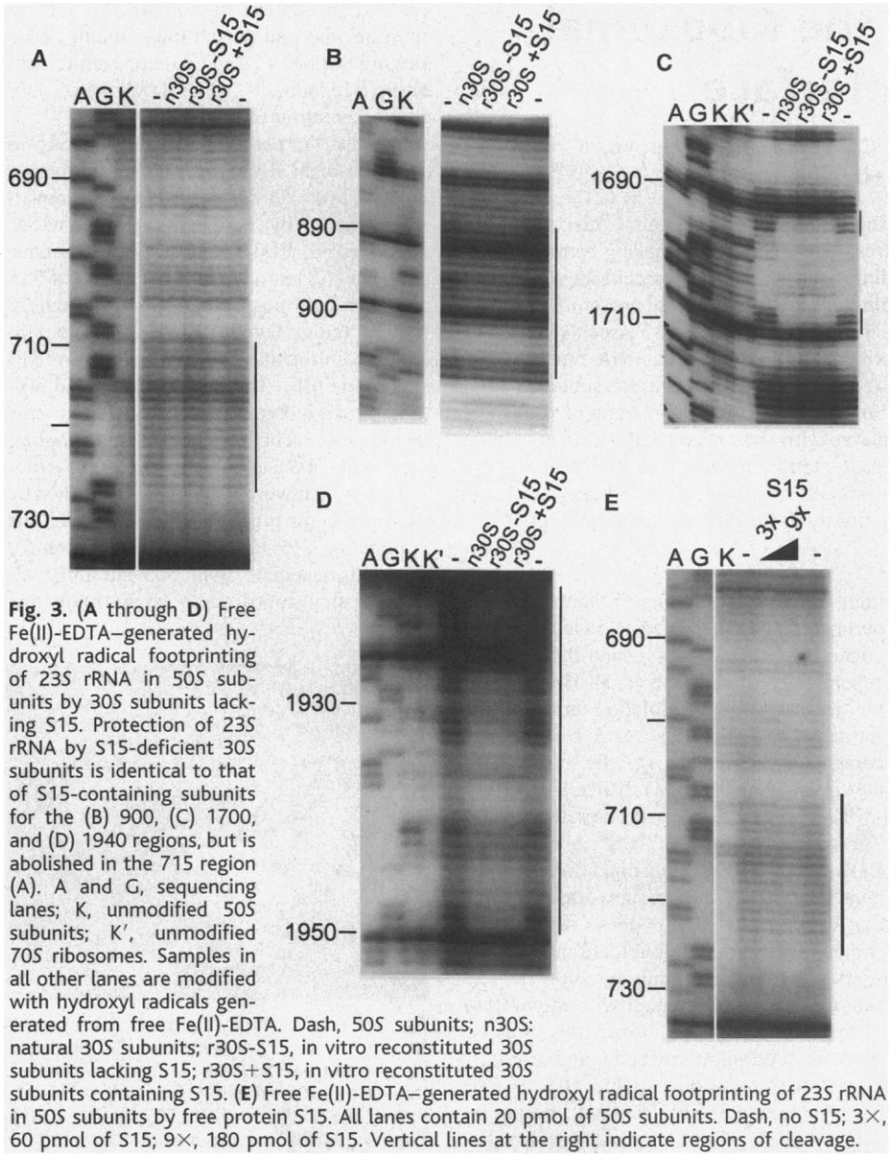

Fig. 3. (**A** through **D**) Free Fe(II)-EDTA–generated hydroxyl radical footprinting of 23*S* rRNA in 50*S* subunits by 30*S* subunits lacking S15. Protection of 23*S* rRNA by S15-deficient 30*S* subunits is identical to that of S15-containing subunits for the (B) 900, (C) 1700, and (D) 1940 regions, but is abolished in the 715 region (A). A and G, sequencing lanes; K, unmodified 50*S* subunits; K′, unmodified 70*S* ribosomes. Samples in all other lanes are modified with hydroxyl radicals generated from free Fe(II)-EDTA. Dash, 50*S* subunits; n30S: natural 30*S* subunits; r30S-S15, in vitro reconstituted 30*S* subunits lacking S15; r30S+S15, in vitro reconstituted 30*S* subunits containing S15. (**E**) Free Fe(II)-EDTA–generated hydroxyl radical footprinting of 23*S* rRNA in 50*S* subunits by free protein S15. All lanes contain 20 pmol of 50*S* subunits. Dash, no S15; 3×, 60 pmol of S15; 9×, 180 pmol of S15. Vertical lines at the right indicate regions of cleavage.

sembled according to other criteria (*12*). This result is also consistent with contact between S15 and the 50*S* subunit. Localized hydroxyl radical production was initiated, and the entire 23*S* rRNA chain was scanned for sites of rRNA cleavage by primer extension (*13*). A single region of 23*S* rRNA, the 715 stem-loop of domain II, was targeted by hydroxyl radicals generated from positions 12 and 46 of S15 (Fig. 2) (*14*). Overlapping cleavage patterns were observed; nucleotides 707 to 711 and 715 to 717 were targeted by Fe(II)-C12-S15 (Fig. 2), and nucleotides 706 and 707 and 716 to 722 were cleaved by Fe(II)-C46-S15 (Fig. 2). No other cleavage was observed in 23*S* or 5*S* rRNA from Fe(II)-S15 (*12*). Placement of the 715 stem-loop at the subunit interface is supported by hydroxyl radical footprinting studies using free Fe(II)-EDTA (*3*), which showed that this same region of domain II became protected upon 70*S* ribosome formation (Fig. 2).

The possibility of direct interaction of S15 with the 715 stem-loop was first tested using footprinting analysis of in vitro reconstituted 30*S* subunits lacking S15 (*15, 16*). Solution hydroxyl radical footprinting (*17*) of the 70*S* particles showed that the 30*S*-dependent protection of the 715 region of 23*S* rRNA was abolished as a result of S15 omission (Fig. 3A), although 30*S*-dependent footprints were unaffected elsewhere in 23*S* rRNA (Fig. 3, B through D). Protection of 23*S* rRNA by 30*S* subunits reconstituted with S15 was indistinguishable from that of natural 30*S* subunits (Fig. 3, A through D).

To further test the possible interaction of S15 with the 715 loop, we bound free S15 protein to 50*S* subunits, and we assessed its interaction with 23*S* rRNA by hydroxyl radical footprinting (*18*). The 715 loop of 50*S* subunits was protected in a concentration-

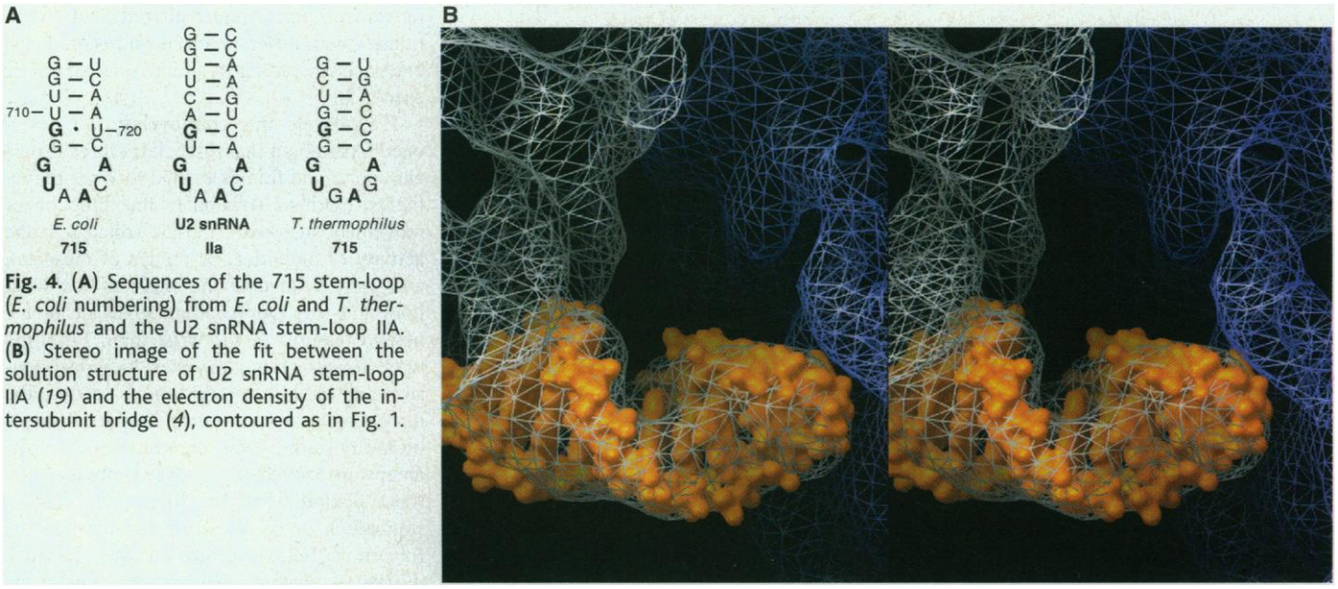

Fig. 4. (**A**) Sequences of the 715 stem-loop (*E. coli* numbering) from *E. coli* and *T. thermophilus* and the U2 snRNA stem-loop IIA. (**B**) Stereo image of the fit between the solution structure of U2 snRNA stem-loop IIA (*19*) and the electron density of the intersubunit bridge (*4*), contoured as in Fig. 1.

dependent manner by S15 in a way that is similar to that observed with intact 30*S* subunits (Fig. 3E). No change in the reactivity of any other 23*S* rRNA nucleotides was observed upon S15 binding, and no change in reactivity of any 23*S* rRNA nucleotides was observed upon binding a mixture of small subunit ribosomal proteins lacking S15 (*12*). These results suggest a specific interaction between ribosomal protein S15 and the 715 stem-loop of 23*S* rRNA. Initial efforts to footprint S15 on naked 23*S* rRNA or on an RNA fragment corresponding to the 715 loop have proven unsuccessful. We conclude that an intersubunit contact is formed by the interaction of protein S15 with the 715 stem-loop of 23*S* rRNA.

There is a striking similarity between the 715 stem-loop of 23*S* rRNA and stem-loop IIA of U2 small nuclear RNA (snRNA), whose solution structure has been determined by nuclear magnetic resonance spectroscopy (*19*). The loop sequences of the *Escherichia coli* 715 loop and U2 loop IIA are identical, whereas that of *T. thermophilus* differs from them in only two positions (Fig. 4A). The close fit of the stem-loop IIA structure to the electron density of the observed bridge (Fig. 4B) provides further confirmation of the identification of this molecular feature of the ribosome interface as the 715 stem-loop of 23*S* rRNA. The arrangement of the loop suggests that nucleotides around the conserved purines at positions 715 and 716 (*E. coli* numbering) make specific interactions with the 30*S* subunit (Fig. 4B). At lower contour levels, electron density from the minor groove side of the 715 stem-loop merges with that of the 30*S* subunit, consistent with the observed hydroxyl radical protection pattern. At the current resolution of the 70*S* ribosome structure, the regions of S15 that participate in this intersubunit contact cannot be unambiguously identified and will require further structural and biochemical studies. It should be possible to use similar combinations of x-ray structural information and biochemical probing analysis to identify additional molecular features of the ribosome and its functional complexes.

References and Notes

1. C. A. Morrison, R. A. Garrett, H. Zeichhardt, G. Stöffler, *Mol. Gen. Genet.* **127**, 359 (1973); T.-T. Sun and R. T. Traut, *J. Mol. Biol.* **87**, 509 (1974); N. M. Chapman and H. F. Noller, *ibid.* **109**, 131 (1977); M. Santer and S. Shane, *J. Bacteriol.* **130**, 900 (1977); W. Herr and H. F. Noller, *J. Mol. Biol.* **130**, 421 (1979); W. Herr, N. M. Chapman, H. F. Noller, *ibid.*, p. 433; J. M. Lambert and R. R. Traut, *ibid.* **149**, 451 (1981); N. Meier and R. Wagner, *Eur. J. Biochem.* **146**, 83 (1985); M. M. Yusupov and A. S. Spirin, *FEBS Lett.* **197**, 229 (1986); U. Scheibe and R. Wagner, *Biochim. Biophys. Acta* **869**, 1 (1986); W. E. Tapprich and W. E. Hill, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 556 (1986); P. Mitchell, M. Osswald, R. Brimacombe, *Biochemistry* **31**, 3004 (1992); C. Merryman, D. Moazed, G. Daubresse, H. F. Noller, *J. Mol. Biol.* **285**, 107 (1999).
2. G. G. Abdurashidova, E. A. Tsvetkova, A. A. Chernyi, L. B. Kaminir, E. I. Budowsky, *FEBS Lett.* **185**, 291 (1985); C. L. Chiam and R. Wagner, *Biochemistry* **22**, 1193 (1983).
3. C. Merryman, D. Moazed, J. McWhirter, H. F. Noller, *J. Mol. Biol.* **285**, 97 (1999).
4. J. H. Cate, M. M. Yusupov, G. Zh. Yusupova, T. N. Earnest, H. F. Noller, *Science* **285**, 2095 (1999).
5. M. Stöffler-Meilecke and G. Stöffler, in *The Ribosome: Structure, Function, and Evolution*, W. E. Hill *et al.*, Eds. (American Society for Microbiology Press, Washington, DC, 1990), pp. 123–133; H. F. Noller *et al.*, unpublished results.
6. W. S. Champney, *Biochim. Biophys. Acta* **609**, 464 (1980).
7. G. M. Heilek, R. Marusak, C. F. Meares, H. F. Noller, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1113 (1995); G. M. Heilek and H. F. Noller, *Science* **272**, 1659 (1996); *RNA* **2**, 597 (1996); G. M. Culver and H. F. Noller, *ibid.* **4**, 1471 (1998); G. M. Culver, G. M. Heilek, H. F. Noller, *J. Mol. Biol.* **286**, 355 (1999); L. Holmberg and H. F. Noller, *ibid.* **289**, 223 (1999); K. R. Lieberman and H. F. Noller, *ibid.* **284**, 1367 (1998); K. Wilson and H. F. Noller, *Cell* **92**, 131 (1998).
8. L. H. DeRiemer, C. F. Meares, D. A. Goodwin, C. J. Diamanti, *J. Labelled Compd. Radiopharm.* **18**, 1517 (1981); T. M. Rana and C. F. Meares, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10578 (1991); J. K. Moran, D. P. Greiner, C. F. Meares, *Bioconjugate Chem.* **6**, 296 (1995).
9. T. A. Kunkel, K. Bebenek, J. McClary, *Methods Enzymol.* **204**, 125 (1991).
10. H. Berglund, A. Rak, A. Serganov, M. Garber, T. Hard, *Nature Struct. Biol.* **4**, 20 (1997); W. M. Clemons Jr., C. Davies, S. W. White, V. Ramakrishnan, *Structure* **6**, 429 (1998).
11. G. M. Culver and H. F. Noller, *RNA* **5**, 832 (1999).
12. G. M. Culver, unpublished observations.
13. ——— and H. F. Noller, *Methods Enzymol.*, in press.
14. Position C36 of S15 was shown to be refractory to derivatization (*12*).
15. Reconstitution of 30*S* subunits was performed as described (*11*), except S15 was excluded from the mixture of group I proteins.
16. The 50*S* subunits and 70*S* ribosomes were purified as described (*11*).
17. For each probing reaction, 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2 mM EDTA (mixed together before addition), 5 mM ascorbate, and 0.05% H_2O_2 (final concentrations) were added to the side of the sample-containing tubes. Probing was initiated by pulse centrifugation at 4°C, and probing was done on ice for 10 min. Reactions were quenched by the addition of 1/10 volume 0.1 M thiourea, and RNA was recovered and analyzed by primer extension as previously described (*13*).
18. Natural 50*S* subunits were incubated alone or in the presence of S15 at 42°C for 1 hour in a total volume of 50 μl, containing 20 mM K^+-Hepes (pH 7.6), 330 mM KCl, and 20 mM $MgCl_2$. RNA probing, isolation, and analysis were done as previously described (*13*).
19. S. C. Stallings and P. B. Moore, *Structure* **5**, 1173 (1997).

20. The authors thank A. Dallas, K. Fredrick, L. Holmberg, and K. Lieberman for comments on the manuscript. This work was supported by NIH grants GM-17129 and GM-59140 (to H.F.N.). J.H.C. is a Damon Runyon–Walter Winchell Postdoctoral Fellow. G.M.C. was supported by NIH postdoctoral fellowship 1F32GM18065-01.

24 August 1999; accepted 2 September 1999

LINKED CITATIONS

Identification of an RNA-Protein Bridge Spanning the Ribosomal Subunit Interface

Gloria M. Culver; Jamie H. Cate; G. Zh. Yusupova; Marat M. Yusupov; Harry F. Noller

Science, New Series, Vol. 285, No. 5436. (Sep. 24, 1999), pp. 2133-2135.
Stable URL:
http://links.jstor.org/sici?sici=0036-8075%2819990924%293%3A285%3A5436%3C2133%3AIOARBS%3E2.0.CO%3B2-Y

References and Notes

[1] **Involvement of Bases 787-795 of Escherichia coli 16S Ribosomal RNA in Ribosomal Subunit Association**

William E. Tapprich; Walter E. Hill

Proceedings of the National Academy of Sciences of the United States of America, Vol. 83, No. 3. (Feb. 1, 1986), pp. 556-560.
Stable URL:
http://links.jstor.org/sici?sici=0027-8424%2819860201%2983%3A3%3C556%3AIOB7OE%3E2.0.CO%3B2-V

[4] **X-Ray Crystal Structures of 70S Ribosome Functional Complexes**

Jamie H. Cate; Marat M. Yusupov; Gulnara Zh. Yusupova; Thomas N. Earnest; Harry F. Noller

Science, New Series, Vol. 285, No. 5436. (Sep. 24, 1999), pp. 2095-2104.
Stable URL:
http://links.jstor.org/sici?sici=0036-8075%2819990924%293%3A285%3A5436%3C2095%3AXCSO7R%3E2.0.CO%3B2-Y

[7] **Directed Hydroxyl Radical Probing of 16S rRNA Using Fe(II) Tethered to Ribosomal Protein S4**

Gabriele M. Heilek; Rosemary Marusak; Claude F. Meares; Harry F. Noller

Proceedings of the National Academy of Sciences of the United States of America, Vol. 92, No. 4. (Feb. 14, 1995), pp. 1113-1116.
Stable URL:
http://links.jstor.org/sici?sici=0027-8424%2819950214%2992%3A4%3C1113%3ADHRPO1%3E2.0.CO%3B2-U

LINKED CITATIONS

[7] **Site-Directed Hydroxyl Radical Probing of the rRNA Neighborhood of Ribosomal Protein S5**
Gabriele M. Heilek; Harry F. Noller
Science, New Series, Vol. 272, No. 5268. (Jun. 14, 1996), pp. 1659-1662.
Stable URL:
http://links.jstor.org/sici?sici=0036-8075%2819960614%293%3A272%3A5268%3C1659%3ASHRPOT%3E2.0.CO%3B2-G

[8] **Transfer of Oxygen from an Artificial Protease to Peptide Carbon During Proteolysis**
Tariq M. Rana; Claude F. Meares
Proceedings of the National Academy of Sciences of the United States of America, Vol. 88, No. 23. (Dec. 1, 1991), pp. 10578-10582.
Stable URL:
http://links.jstor.org/sici?sici=0027-8424%2819911201%2988%3A23%3C10578%3ATOOFAA%3E2.0.CO%3B2-K